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Review

Neurotransmitter profiles in fish gills: Putative gill oxygen chemoreceptors☆

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ABSTRACT

In fish, cells containing serotonin, ACh, catecholamines, NO, H_2S, leu-5-enkephalin, met-5-enkephalin and neuropeptide Y are found in the gill filaments and lamellae. Serotonin containing neuroepithelial cells (NECs) located along the filament are most abundant and are the only group found in all fish studied to date. The presence of NECs in other locations or containing other transmitters is species specific and it is rare that any one NEC contains more than one neurochemical. The gills are innervated by both extrinsic and intrinsic nerves and they can be cholinergic, serotonergic or contain both transmitters. Some NECs are presumed to be involved in paracrine regulation of gill blood flow, while others part of the reflex pathways involved in cardiorespiratory control. There is both direct and indirect evidence to indicate that the chemosensing cells involved in these latter reflexes sit in locations where some monitor O_2 levels in water, blood or both, yet the anatomical data do not show such clear distinctions.

1. Introduction

In mammals, the carotid body is a multimodal organ responsible for sensing changes in blood PO_2, PCO_2, pH, as well as temperature, glucose, and $[K^+]$ amongst other variables (reviewed by Kumar et al., 2009). In response to a drop in blood PO_2 the glomus cells (the primary oxygen sensing cells in the carotid body) release neurotransmitters onto afferent nerve fibres of the carotid sinus nerve. This nerve is part of the glossopharyngeal (IXth cranial) nerve, which projects centrally to the nucleus tractus solitarius (NTS) in the central nervous system. Here, peripheral chemoreceptor information is integrated and in turn modulates the output from the central respiratory and cardiovascular centres. This gives rise to cardio-respiratory responses that act to match oxygen supply to oxygen demand by the body. Glomus cells not only release neurotransmitters onto afferent nerve fibres, but also receive reciprocal synapses from the afferent nerve fibres as well as efferent innervation from parasympathetic autonomic nerves (reviewed by Campanucci and Nurse, 2007). Much less is known about the less studied reciprocal afferent and efferent innervation of the carotid body but evidence suggests that they modulate the response of glomus cells to hypoxia.

Initially, glomus cells, as well as other oxygen sensing cells such as the chromaffin cells found in the adrenal medulla of mammals, were characterized as amine precursor uptake and decarboxylation (APUD) cells (Pearse et al., 1973). This characterization indicated that these cells contain serotonin, catecholamines, and/or their precursor amines and it was further determined that these cells develop from neural crest cells. Since their early characterization, the glomus cells have been shown to contain as many neurochemicals as the brain, despite the small size of the carotid body (Kumar et al., 2009).

Presently, acetylcholine (ACh) and ATP are thought to be the primary neurotransmitters in the carotid body in most mammals involved in the acute hypoxic response (Nurse, 2005, 2010). Furthermore, one of the most common labels used for identifying glomus cells has been the presence of catecholamines (Pearse et al., 1973). Catecholamines are released from the carotid body during hypoxia and are thought to be involved in mediating the hypoxic response by their interaction with ACh. Low doses of dopamine (DA) enhance the response to ACh, but high doses of DA inhibit the response to ACh (Zapata et al., 2000). Norepinephrine also has excitatory and inhibitory effects on carotid body function (Lahiri et al., 2006). Serotonin is present in glomus cells and its role in the hypoxic response remains unclear but might involve modulation of carotid body sensitivity acting via receptors at pre- and post-synaptic locations in both autocrine and paracrine fashion (Nurse, 2010). Carotid bodies contain many other neurochemicals that modulate carotid body responses to hypoxia and these have been thoroughly reviewed elsewhere (Lahiri et al., 2006).

In fish, the putative oxygen sensing cells are neuroepithelial cells (NECs) found dispersed throughout the gill arches of all fish studied to date (Coolidge et al., 2008; Jonz et al., 2004; Saltys et al.,

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2006). Although similar in many ways to the neuroepithelial bodies found in the lungs of terrestrial vertebrates, NECs are solitary rather than organized in clusters as is the case in mammalian lungs. Many parallels have been drawn between fish NECs and mammalian glomus cells due to similarities in embryonic origin, innervation and response to hypoxia (Jonz and Nurse, 2009; Milsom and Burleson, 2007). Glomus cells and NECs share similar cell ultrastructure, both being mitochondrion rich and containing numerous dense core vesicles. They also share similar innervation patterns: the glomus cells are innervated by the glossopharyngeal nerve while the NECs are innervated by both the glossopharyngeal and vagus nerves (and in some cases the facial nerve). Both NECs and glomus cells also respond to hypoxia by depolarizing and this response involves the closing of a background K^+ channel (Buckler, 2007; Jonz et al., 2004).

Despite all these similarities, most recent research in fish has focused on the one neurotransmitter in mammals least involved in the response to hypoxia – serotonin. Other neurotransmitters have been implicated in oxygen chemosensing in fish gills however, and the purpose of this review is to outline the distribution of various neurochemicals in putative oxygen gill chemoreceptors in fish, as well as to identify research areas that still remain to be explored to further our understanding of their roles in oxygen chemoreception in fish.

2. Serotonin

2.1. Distribution of serotonin in the gills

NECs in fish gills were first described in the early 1980s in trout (*Oncorhynchus mykiss*), perch (*Perca flaviatilis*), pike perch (*Stizostedion lucioperca*), black bass (*Micropterus dolmieu*), black bullhead (*Ictalurus melas*), eel (*Anguilla anguilla*) and shark (*Scyliorhinus canicula*) (Dunel-Erb et al., 1982). They were found in the gill filaments, contained dense cored vesicles and lay in close proximity to nerve terminals. In the same study it was shown that in trout exposed to 30 min of hypoxia (<10 torr), the dense cored vesicles degranulated and there was a decrease in dense core vesicle density (i.e. neurotransmitter was released in response to hypoxia). It was subsequently shown that these cells contained serotonin (Bailly et al., 1992) as do, polymorphous granular cells (PGCs) (Bailly et al., 1989, 1992) and neurons (Bailly et al., 1989).

More recent studies have confirmed this distribution of NECs in the filaments of fish using a combination of immunohistochemistry and confocal imaging in zebrafish (*Danio rerio*), trout (*O. mykiss*), goldfish (*Carasius auratus*), traira (*Hoplias malabaricus*), trairaõ (*Hoplias lacerdae*), and mangarove rivulus (*Kryptolebias marmoratus*) (Coolidge et al., 2008; Jonz et al., 2004; Saltys et al., 2006; Tzaneva and Perry, 2010; Zhang et al., 2011), as well as in sockeye salmon (*Oncorhynchus nerka*) and medaka (*Oryzias latipes*) (Brink and Milsom, unpublished data) (Fig. 1). These cells contain serotonin, are relatively large (7–10 μm in diameter) and are found in the filament epithelium near the efferent filament artery (Jonz et al., 2004). They are usually found along the entire length of the filament, but are more concentrated towards the distal half of the filament (Fig. 1), especially in medaka where this distribution is exaggerated (Fig. 1D).

In more recent studies using confocal microscopy, serotonergic NECs have also been found in the lamellae of all species mentioned above except trout (Coolidge et al., 2008; Jonz et al., 2004; Saltys et al., 2006; Zhang et al., 2011) (Fig. 1) and mangrove rivulus (Regan et al., 2011). The NECs in the lamellae are about half the size of the ones found in the filament and are concentrated towards the tips of the lamellae. In goldfish (*C. auratus*) exposed to warm and/or hypoxic water, when the interlamellar mass is reduced, these NECs are found all along the lamellae. When goldfish are exposed to cold and/or normoxic water, the NECs relocate to the tips of the lamellae as the interlamellar mass fills the space between adjacent filaments (Tzaneva et al., 2011; Tzaneva and Perry, 2010) (Fig. 2).

Additionally, serotonergic cells have also been found in the epithelium surrounding the gill rakers of trout and goldfish. In gold-

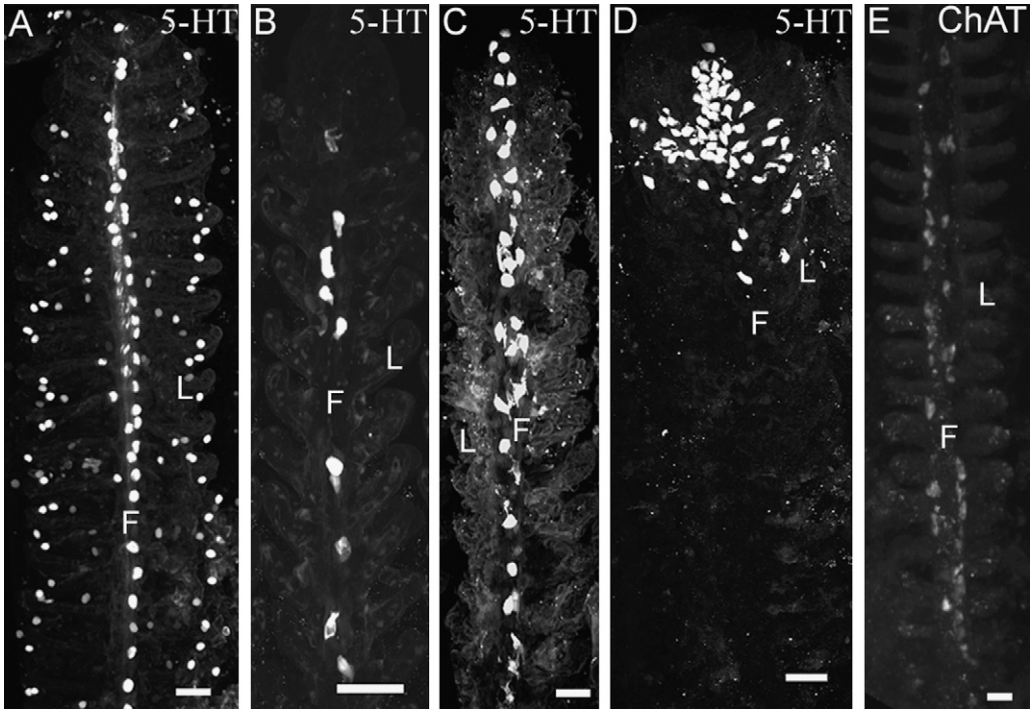

Fig. 1. Distribution of serotonin and acetylcholine in various species of fish. Serotonergic neuroepithelial cells (NECs) in the filament and lamellae of goldfish (*Carasius auratus*) (A), sockeye salmon (*Oncorhynchus nerka*) (B), trout (C) and medaka (*Oryzias latipes*) (D). Choline acetyltransferase (ChAT) labels cells in the vicinity of the location of mitochondrion rich cells in goldfish (*C. auratus*) (E). Scale bars, 50 μm in A, and 20 μm for B–E. Tissues were prepared for immunohistochemistry and imaged using confocal microscopy following procedures similar to Jonz et al. (2004). F, filament; L, lamellae.

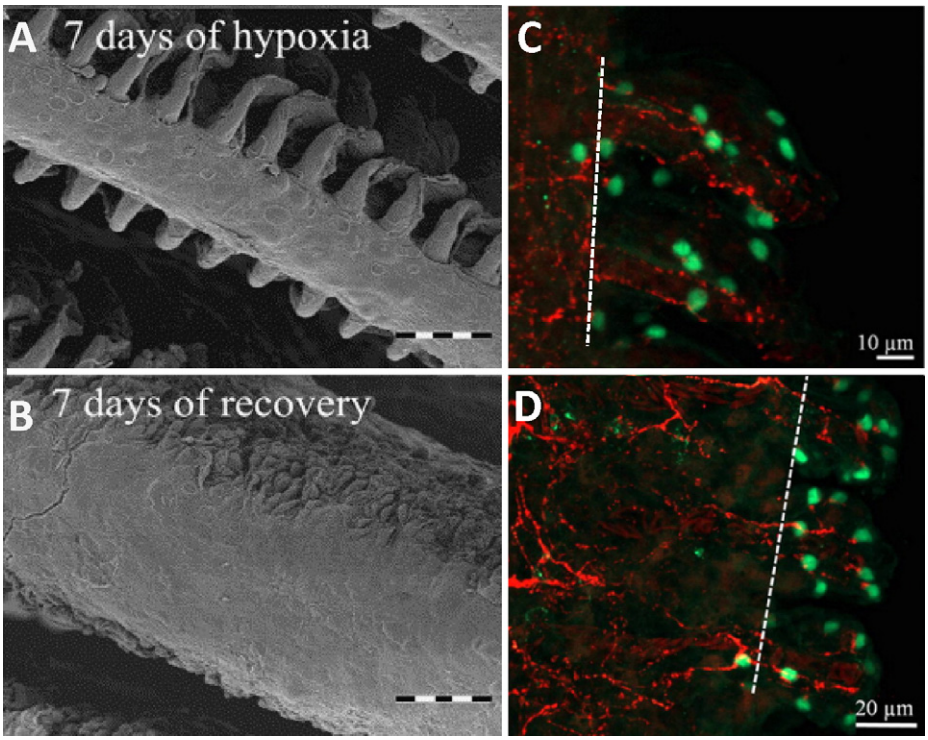

Fig. 2. The effect of gill remodelling during hypoxia on the distribution of lamellar neuroepithelial cells (NECs) in crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*). (A) Scanning electron micrographs from the crucian carp gill arch after 7 days of exposure to hypoxia showing protruding lamellae. (B) Scanning electron micrographs from the crucian carp gill arch after 7 days of recovery in normoxia after hypoxic exposure showing the embedded lamellae (covered with interlamellar cell mass). (C) Confocal micrographs showing serotonergic NECs (green) and their innervation using a neuronal marker (zn-12, red) in the lamellae of goldfish acclimated to warm water (25 °C). (D) Confocal micrographs showing serotonergic NECs (green) and their innervation using a neuronal marker (zn-12, red) in the lamellae of goldfish acclimated to cold water (7 °C). Dashed white line represents the edge of the interlamellar cell mass. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

A and B reprinted with permission from Sollid et al. (2003). C and D reprinted with permission from Tzaneva and Perry (2010).

fish their morphology resembles that of lamellar NECs; however, in trout these cells did not contain synaptic vesicles indicating that perhaps they are not NECs (Coolidge et al., 2008). In zebrafish (*D. rerio*), Merkel-like cells containing serotonin have been described at the tips of the gill rakers suggestive that these cells play a chemical or mechanical sensory role (Zachar and Jonz, 2012). Thus, at present, although serotonergic cells have been found in gill rakers of all of these species, their morphologies and immunohistochemical labelling suggest they may not all be of the same cell type.

Serotonin has also been found in PGCs of trout (*O. mykiss*) but not other species. In trout these cells are found deeper within the filament, at the level of the central venous sinus (Bailly et al., 1989). Although similar in size to the NECs found on the efferent surface of the filaments, PGCs are more elongated and irregular in shape. Because of their position, sitting on either side of the central venous sinus, they often appear as a double row along the filament. These cells are also innervated (Bailly et al., 1989).

2.2. Role in oxygen chemoreception

Support for the involvement of serotonin containing NECs in eliciting cardiorespiratory reflexes comes from electrophysiological studies in which single nerve fibres in isolated gills of trout (*O. mykiss*) that responded to either internal or external hypoxia were also excited when the gills were perfused with serotonin (Burleson and Milsom, 1995) (Fig. 3). These afferent nerve fibres also responded to dopamine, and exhibited a particularly brisk response to similar concentrations of ACh (see below). More recently, patch clamp experiments on large isolated serotonergic NECs, from zebrafish, presumably from the efferent edge of the gill filaments, indicated that these cells depolarize in response to hypoxia and that this response involves a background potassium channel (Jonz et al., 2004 as described elsewhere in this issue). This work suggests that these cells behave very much like the oxygen sensing glomus cells of the mammalian carotid body (López-Barneo et al., 2001). Unfortunately these studies did not confirm that serotonin was released on depolarization.

Carotid body glomus cells undergo cell proliferation and hypertrophy when mammals are exposed to sustained hypoxia (a few days to a month) (Wang et al., 2008) and it has been proposed that this is associated with increased neurochemical turnover (McGregor et al., 1984). Surprisingly, in both zebrafish (*D. rerio*) and mangrove rivulus (*K. marmoratus*) exposed to sustained hypoxia (28 days and 7 days respectively) there was an increase in serotonergic NEC cell size (hypertrophy) but not density (hyperplasia) (Jonz et al., 2004; Regan et al., 2011; Vulesevic et al., 2006) (Fig. 4). However, zebrafish exposed to hyperoxia for 60 days did exhibit a lower serotonergic NEC density than control fish (Vulesevic et al., 2006). These findings generally support a role for serotonergic NECs in oxygen sensing.

Further support comes from behavioural observation on the mangrove rivulus (*K. marmoratus*). This fish responds to acute severe hypoxia by emersion from water and uses its moist skin to obtain oxygen on land. Pre-exposure to serotonin caused these fish to emerse at a higher oxygen tension, while pre-exposure to ketanserin, a serotonin receptor antagonist, caused them to emerse at a lower oxygen tension (Regan et al., 2011) (Fig. 3G). The change in behaviour is thought to be induced by serotonergic NECs in the gill filament and/or skin in these fish.

To date there is no support for a role of other serotonin containing cells in eliciting cardiorespiratory reflexes. The migration of the lamellar NECs in goldfish during restructuring of the gill

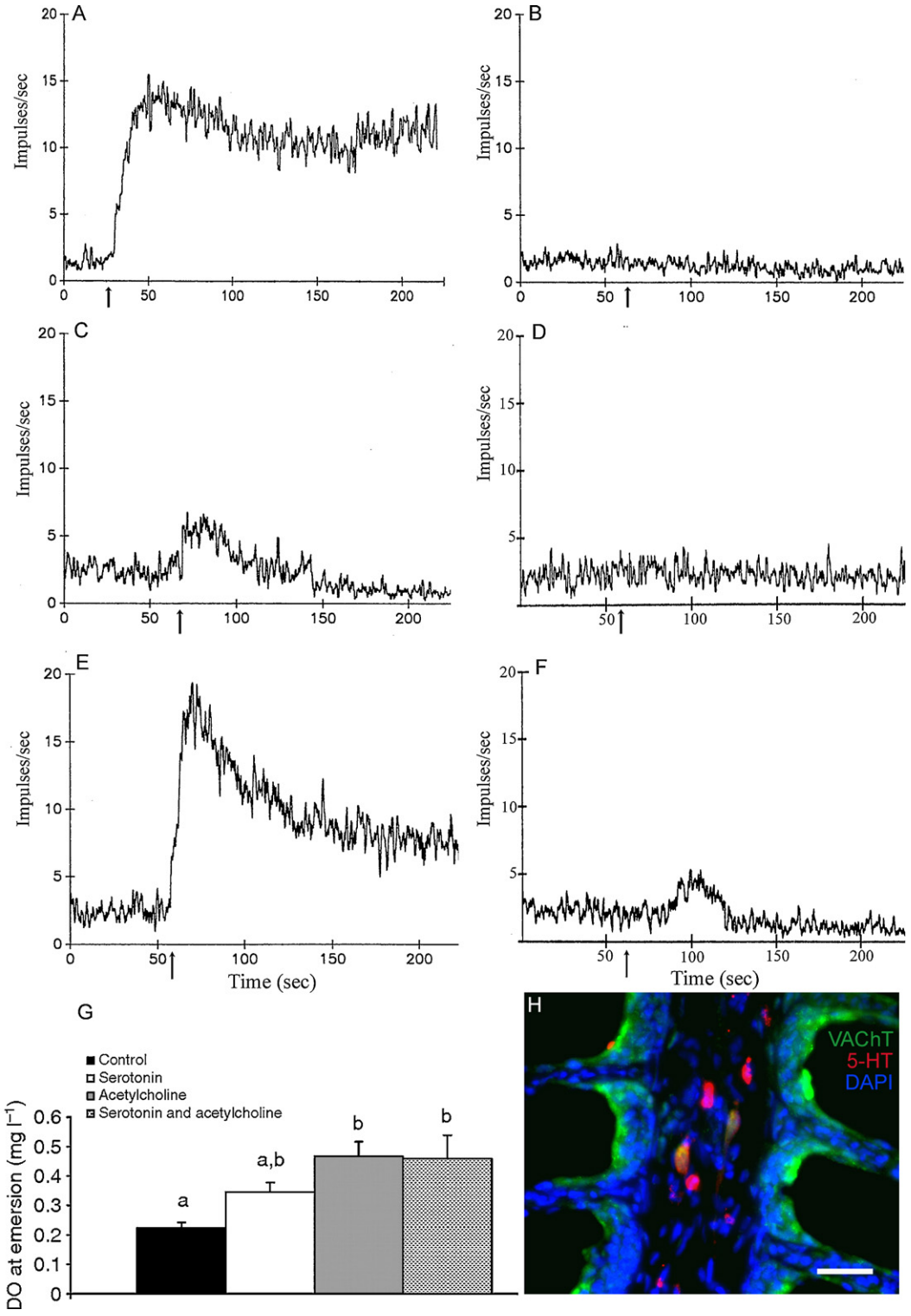

Fig. 3. The effect of various neurochemicals on the afferent nerve discharge of trout (*Oncorhynchus mykiss*) and the emersion behaviour of mangrove rivulus (*Kryptolebias marmoratus*). Mean response of afferent nerve discharge to perfusion with various chemicals: (A) sodium cyanide (NaCN, 25 μg); (B) epinephrine (EPI, 500 nmol); (C) serotonin (5-HT, 100 nmol); (D) norepinephrine (NE, 100 nmol); (E) acetylcholine (ACh, 100 nmol); (F) dopamine (DOPA, 100 nmol). (G) The level of dissolved oxygen (DO) in which emersion behaviour took place in control fish, and those pre-treated with either 1 $\mu mol\,l^{-1}$ serotonin, 1 $mmol\,l^{-1}$ acetylcholine and both serotonin and acetylcholine for 10 min. Means not sharing the same letters are significantly different than one another ($p < 0.05$). (H) Triple immune-labelling of bowfin vesicular acetylcholine transporter (VAChT, green), serotonin (5-HT, red) and cell nuclei (DAPI, blue) in bowfin, *Amia calva*. Scale bar, 50 μm. Tissues were prepared following procedures similar to Porteus et al. (in press). Arrows indicate the time of the addition of the drug.

A, C, E, F is reprinted from Burleson and Milsom (1995), B and D from Burleson (1991), G from Regan et al. (2011) with permission.

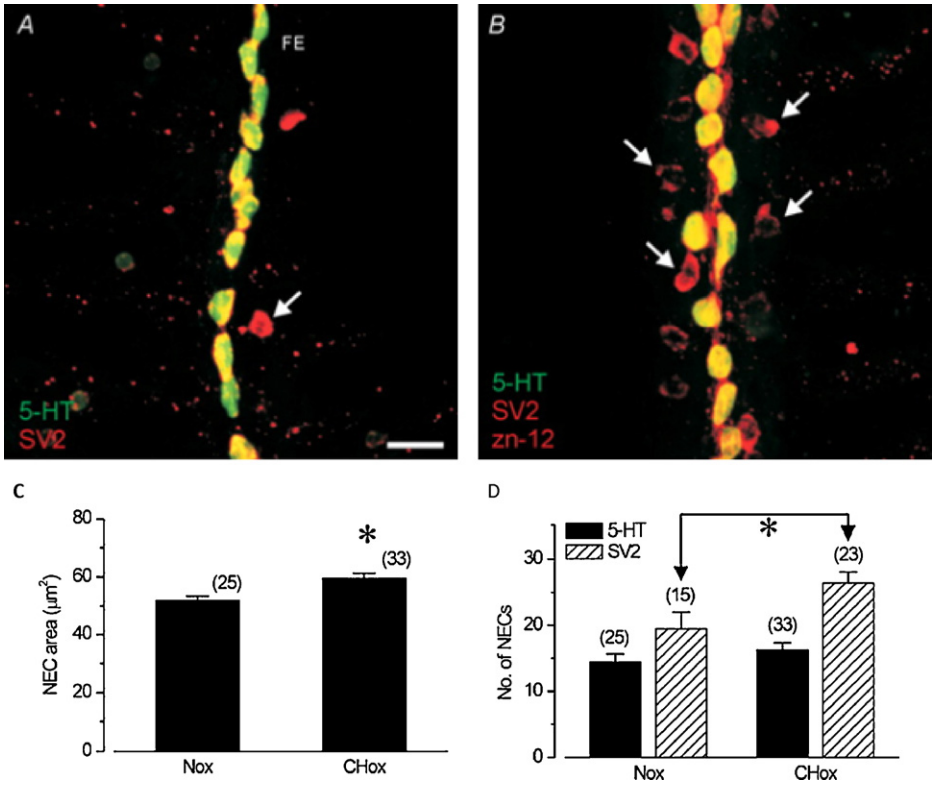

Fig. 4. The effect of hypoxia on NECs morphology in zebrafish (*Danio rerio*) exposed to 28 days of hypoxia. (A) NECs labelled with serotonin (5-HT, green) and a synaptic vesicle marker (SV2, red) in a zebrafish exposed to normoxia. Scale bar, 20 μm. (B) The NECs in a zebrafish after exposure to 28 days of hypoxia showing an increase in cell size in NECs in the filament. (C) Mean size (μm^2) of NECs from normoxia and hypoxia exposed fish. (D) Mean density (# of cells) of 5-HT+ NECs and total NECs including 5-HT+ (labelled with SV2). *Significant differences ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Reproduced with permission from Jonz et al. (2004).

would imply their position in contact with the water is important (Tzaneva and Perry, 2010). However, lamellar NECs are not found in all species (Coolidge et al., 2008) and in zebrafish, hypoxic hyperventilation appears early in development before the formation of the filamental lamellae (Saltys et al., 2006). Given their small size, patch clamp recordings have not been obtained from these cells. There is also no evidence to date to support a role of the NECs on the gill rakers, or of the PGCs in cardiorespiratory reflexes.

Confocal imaging and labelling with neuronal markers have shown that most of the serotonergic NECs in the filament and those in the lamellae receive innervation arising from outside the gill filament (extrinsic) likely in the central nervous system (Jonz and Nurse, 2003). These nerves could be afferent or efferent. They could be part of the reflex pathway giving rise to cardiorespiratory responses to hypoxia, or they could be part of an efferent mechanism either modulating the afferent pathways or regulating blood flow (see Bailly, 2009 for review). The NECs in the filament also receive innervation from nerves that have cell bodies within the gill arch (intrinsic) that project to the sphincter region of the efferent filament artery (eFA) (Jonz and Nurse, 2003). Interestingly, some of these nerves are serotonergic. Three types of intrinsic neurons have been described in zebrafish, goldfish and trout: chain neurons, deep proximal neurons and superficial proximal neurones (Jonz and Nurse, 2003; Porteus et al., in press) (Fig. 5A). The chain neurons are evenly distributed and lie within the filament, near the efferent filament artery but do not innervate NECs. Deep proximal neurons are found near the base of the efferent filament artery and project distally near this blood vessel but do not innervate NECs. Superficial proximal neurons also originate near the base of the efferent filament artery and travel distally along this vessel and do innervate NECs (Fig. 5A). It is has been suggested that NECs can act by releasing neurochemicals on superficial proximal neurons in response to hypoxia, which then act on the muscle of the eFA, causing it to constrict and alter blood pressure and flow through the gill (Jonz and Nurse, 2003; Sundin et al., 1995).

2.3. Vasomotor role of serotonin

Serotonin has also been shown to be a potent vasoconstrictor in fish gills (Fritsche et al., 1992; Östlund and Fänge, 1962; Reite, 1969). Support for the involvement of serotonin containing NECs (both filamental and lamellar) in paracrine regulation of blood flow comes from studies looking at their location and serotonergic innervation as well as the effect of exogenous serotonin injections on gill blood flow. As just described, proximal neurons containing serotonin innervate the efferent filament artery sphincter (Jonz and Nurse, 2003) and lamellar NECs are present near pillar cells which do contract and redistribute blood flow (Stensløkken et al., 2006). However, it is not clear whether serotonin is actually released into the circulation during hypoxia nor how it acts in combination with circulating catecholamines and/or the cholinergic innervation of the efferent filament artery sphincter to modulate oxygen uptake at the gills.

Clearly serotonin containing NECs are ubiquitous but differentially distributed in different fish species. The relative roles of the various NECs and the reasons for species differences in their distribution remain unclear. While some serotonergic NECs are possibly involved in eliciting cardiorespiratory reflexes, others likely play a paracrine role in regulating gill blood flow. Much remains to be discerned. It should also be noted that NECs (as identified by labelling for the synaptic vesicle marker SV2), not containing serotonin, undergo hyperplasia in zebrafish following sustained hypoxia. This

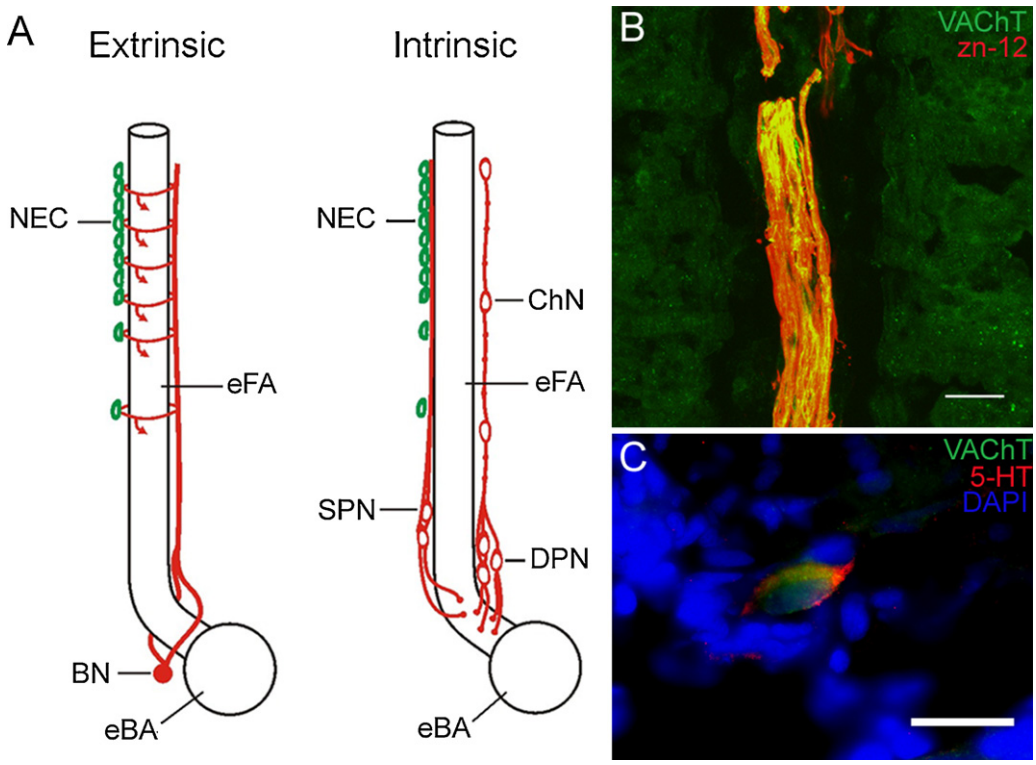

Fig. 5. Proposed innervation for gill serotonergic neuroepithelial cells (NECs) by cholinergic nerve fibres and neurons. (A) Proposed innervation pattern of the zebrafish gill of serotonergic NECs. Extrinsic nerves (bundle neurons, BN) innervate NECs and project to the central nervous system. Superficial proximal neurons (SPN) also innervate NECs but project to the efferent filament artery (eFA) sphincter area near the efferent branchial artery (eBA). Deep proximal neurons (DPN) innervate serotonergic chain neurons (ChN) but not NECs and also project to the eFA sphincter area. Reproduced with permission from Jonz and Nurse (2003). (B) Z-stack compression of double immunolabelling of vesicular acetylcholine transporter (VAChT) and a neuronal marker (zn-12) in the extrinsic innervation of goldfish (*Carassius auratus*) first gill arch. (C) Wide-field image of triple immune-labelling of a proximal neuron with VAChT (green), serotonin (5-HT, red) and cell nucleus marker (DAPI, blue) of trout (*Oncorhynchus mykiss*). Scale bars, 20 μm in B and 10 μm in C. Tissue was prepared for immunohistochemistry, and imaged by confocal microscopy following procedures similar to Porteus et al. (in press). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

is suggesting that either these cells are precursor NECs or that they are NECs containing an as yet unidentified neurochemical also contribute to O_2 chemosensing (Jonz et al., 2004) (Fig. 4) and we explore these possibilities in the remainder of this article.

3. Acetylcholine

ACh appears to be the main neurotransmitter in the carotid body involved in the acute response to hypoxia in mammals (Zapata, 2007), and appears to play a role in O_2-sensing in fish, but exactly how it does so remains uncertain. Electrophysiological recording from isolated gills of trout (*O. mykiss*) demonstrated that single nerve fibres sensitive to hypoxia and NaCN were also strongly stimulated by ACh and nicotine (Fig. 3) (Burleson and Milsom, 1995). Furthermore, the emersion response in the facultative air-breathing mangrove rivulus, *K. marmoratus*, was accentuated in fish pre-exposed to ACh and attenuated in fish pre-exposed to the nicotinic antagonist, hexamethonium (Regan et al., 2011) (Fig. 3G).

Attempts to identify ACh containing NECs, however, have shown that NECs do not contain this neurochemical. Immunohistochemical labelling for markers for vesicular acetylcholine transporter (VAChT) and synaptic vesicles (SV2) have not shown any NECs containing ACh in trout (*O. mykiss*), goldfish (*C. auratus*) (Porteus et al., in press) or mangrove rivulus (*K. marmoratus*) (Regan et al., 2011). However, in goldfish (*C. auratus*) one population of cells labelling for choline acetyltransferase (ChAT) and SV2 have been found in the interlamellar spaces (Fig. 1E) (Brink and Milsom, unpublished data). These cells sit in close proximity to the mitochondrial rich cells between the central venous sinus and the epithelium but do not appear to be innervated. VAChT (vesicular ACh transporter) containing cells have also been described in bowfin (Porteus and Milsom, unpublished) and mangrove rivulus (Regan et al., 2011). Those in the bowfin are located in the epithelium between lamellae (Fig. 3H). While it remains possible that ACh containing NECs are present in all fish species but have not been identified due to a lack of appropriate antibodies for immunohistochemistry, there is another parsimonious explanation of the electrophysiological and emersion data presented above.

Populations of neurons that label for vesicular acetylcholine transporter (VAChT) have also been identified in trout and goldfish (Porteus et al., in press) and are similar to the chain and proximal neurons previously described in zebrafish (Jonz and Nurse, 2003). Additionally, acetylcholine has been found in nerve fibres running in the nerve bundle of the gill as part of the extrinsic innervation (Porteus et al., in press). Thus, another explanation of the electrophysiological and emersion data is that ACh is released at a synapse somewhere in the gills (the electrophysiology was performed on excised gills) that is part of the reflex pathway. There are several sites at which this could occur. The first is via the reciprocal synapses between the NECs and their afferent innervation (Dunel-Erb et al., 1982). In this scenario a neurotransmitter (such as serotonin) released by the NEC in response to hypoxia would cause the release of ACh from the afferent nerve terminal. This in turn would act in a positive feedback manner on the NEC to cause more release of neurotransmitter by the NEC enhancing afferent nerve discharge. ACh might also be released from efferent nerves onto NECs to enhance their transmitter release and stimulate afferent nerve discharge. Reciprocal synapses have been well described between the glomus cells and their afferent innervation in the carotid bodies in mammals (Shirahata et al., 2007) as has

innervation of glomus cells by efferent nerves that contain ACh that modulates the response of glomus cells to hypoxia, either directly or via nitric oxide (Campanucci and Nurse, 2007). Furthermore, two types of innervation of NECs have been described in catfish (*I. melas*), one containing dense cored vesicles and one containing smaller clear vesicles, typical of cholinergic innervation (Dunel-Erb et al., 1982). Consistent with these scenarios, when *K. marmoratus* were pre-exposed simultaneously to the neurotransmitters serotonin and acetylcholine, the emersion response was not significantly different from trials where the fish were exposed to either drug alone (Regan et al., 2011) (Fig. 3G). While this could be accounted for by saturation of the response, it would also occur if serotonin- and acetylcholine-containing cells were elements in a common pathway.

ACh has been also shown to have effects on the vasomotor tone of fish gills. Cholinergic nerve fibres innervate the efferent filament sphincter in trout (*O. mykiss*), eel (*A. anguilla*), cod (*Gadus morhua*), perch (*Perca fluviatilis*) (Bailly and Dunel-Erb, 1986), and goldfish (Porteus et al., in press) and ACh injections into the branchial vasculature of trout cause a general vasoconstriction of the efferent filament artery and arterioles (for reviews see Olson, 2002; Sundin and Nilsson, 1997). Thus, stimulation of cholinergic nerves causes an increase in branchial resistance and a decrease in blood flow to the distal portions of the gill filaments. A reduction in flow has been shown previously to increase nerve discharge in afferent fibres in isolated gills of yellowfin tuna, *Thunnus albacares* (Milsom and Brill, 1986) suggesting that a decrease in blood flow to the distal parts of the filament might reduce O_2 delivery and indirectly stimulate oxygen chemoreceptors found in that area of the gill.

Finally, in *K. marmoratus*, the blockade of muscarinic receptors with atropine increased the emersion response to hypoxia (Regan et al., 2011). It was suggested that stimulation of muscarinic receptors might inhibit the release of acetylcholine during hypoxia, similar to the situation found in the central nervous system of mammals (Carey et al., 2001).

In summary to this section, the source and role of ACh in O_2-chemosensing in fish remains enigmatic. It is clear that ACh is involved in this response but determining just how is fruit for much further investigation.

4. Catecholamines

As mentioned earlier, glomus cells and NECs in mammals are characterized as amine precursor uptake and decarboxylation (APUD) cells (Pearse et al., 1973) not only containing serotonin, but also catecholamines, and/or their precursor amines. Initial experiments on fish NECs using formaldehyde-induced florescence determined that the only monoamine found in these cells was serotonin (Dunel-Erb et al., 1982). Application of parachlorophenylalanine, a serotonin inhibitor, abolished the formaldehyde-induced florescence indicating the absence of other monoamines such as catecholamines. More recent studies have been equivocal.

Non-serotonergic NECs, which increase in number following chronic hypoxic exposure, have been reported previously in several fish species (Coolidge et al., 2008; Jonz et al., 2004; Jonz and Nurse, 2003) and it is thought that they are either precursor NECs or that they might contain other neurochemicals, such as catecholamines. Most studies using immunolabelling for the synthetic enzyme of catecholamines, tyrosine hydroxylase, however, have failed to find evidence for the presence of catecholamines in NECs in trout, goldfish (Porteus et al., in press) or catfish (*Heteropneustes fossilis*) (Zaccone et al., 2003). Additionally, western blot analysis showed an absence of tyrosine hydroxylase in the gills of barramundi (*Lates calcarifer*) (Candy and Collet, 2005). Consistent with these findings, epinephrine and norepinephrine failed to alter the discharge profiles of single afferent nerve fibre in isolated gills from trout although dopamine produced a weak excitation followed by a weak inhibition of nerve discharge (Burleson and Milsom, 1995) (Fig. 3D–F). However, NECs immunopositive for tyrosine hydroxylase have been found in cultured cells from channel catfish (*Ictalurus punctatus*) (Burleson et al., 2006). Patch clamp recordings from these latter cells revealed populations of cells with two different responses to hypoxia: one that was inhibited (hyperpolarized) by hypoxia, and one population that was excited (depolarized) by hypoxia.

As with serotonin and ACh, catecholamines also play an important role in regulating gill blood flow during hypoxia. There is sympathetic innervation of the anastomoses between the arterio-arterial and arterio-venous circulations in fish gills, as well as some scarce innervation at the base of the filaments around the efferent filament artery sphincter but the role of this innervation is still unclear (Dunel-Erb and Bailly, 1986; Nilsson and Sundin, 1998). Furthermore, stimulation of NECs in the gills by severe hypoxia increases levels of circulating catecholamines released from the chromafin cells in the head kidney (Reid and Perry, 2003) that enhance oxygen uptake at the gills. A vasoconstriction caused by adrenaline on the efferent filament artery that is counteracted by a vasodilation of the afferent filament artery resulting in no change in arterio-arterial blood pressure, but an increase in arterio-arterial functional surface and thus, oxygen uptake (Sundin and Nilsson, 2002).

5. ATP

In mammals, ATP is released along with ACh from the glomus cells of the carotid body in response to hypoxia (Zhang et al., 2000). Immunolabelling for purinergic receptors in zebrafish revealed $P2X_3$ receptors in the lamellae in a subset of serotonergic NECs that had a morphology that was distinct from that of other serotonergic NECs found in the lamellae (Jonz and Nurse, 2003). However, these cells were not innervated indicating that these cells could not be involved in cardiorespiratory reflex responses. ATP is ubiquitous and thus, is probably produced by nearby cells and acts on $P2X_3$ receptors on the serotonergic NECs to induce the release of this vasoactive substance. As such it likely plays a paracrine role in regulating lamellar blood flow.

6. Nitric oxide (NO)

In the past two decades research has focused on the role of gasotransmitters in cardiovascular function in both fish and mammals (reviewed by Olson and Donald, 2009). Nitric oxide (NO) is one of these gasotransmitters and it is generated by the enzyme nitric oxide synthase (NOS). In mammals, 3 isoforms of the enzyme exist: brain or neuronal NOS (nNOS), inducible NOS (iNOS or NOS II) and endothelial NOS (eNOS) and the distribution in animals tissues is implied by the isoform name. In mammals, NO is co-released with ACh to cause vasodilation of blood vessels.

In fish, only the first two isoforms of NOS have been identified: nNOS and iNOS (Hyndman et al., 2006). Of these, nNOS has been found in some cells in the filaments of channel catfish (*I. punctatus*) but it is unknown whether these also contained other neurotransmitters (Zaccone et al., 2003). In this study immunolabelling with nNOS antibodies revealed nitrergic nerve fibres innervating the efferent filament artery walls, mucous cells in the filament, pillar cells in the lamellae, and intralamellar cells (Zaccone et al., 2003). While NO produces vasoconstriction or no effect on blood vessels (reviewed by Olson and Donald, 2009) exposure of catfish to hypoxia (75 torr for 7 days) has been shown to produce an increase

in the density of nitrergic nerve fibres indicating a role of NO in the hypoxic response (Zaccone et al., 2006).

7. Hydrogen sulphide (H_2S)

Another gasotransmitter that has received recent attention is hydrogen sulphide (H_2S). H_2S was first identified as a regulator of vascular tone in both fish and mammals (Hosoki et al., 1997; Olson et al., 2006) and has been proposed as a potential oxygen sensor in the mammalian renal medulla, the chromaffin cells of trout, and the carotid body (Olson, 2011; Olson et al., in press). In trout (*O. mykiss*), the H_2S synthesizing enzymes have been found in gill homogenates and in hypoxia, gill tissue produces H_2S in vitro. Furthermore, injection of a bolus of H_2S into the ventral aorta produces a bradycardia and ventilatory response similar to that induced by hypoxia. The removal of the first gill arch eliminates the heart rate response but not the ventilatory response during hypoxia. In zebrafish (*D. rerio*), isolated NECs also depolarize in response to high levels of H_2S (Olson, 2008). It is hypothesized that H_2S is continuously produced in the cell from the amino acid cysteine in a reaction catalyzed by one of the enzymes cystathionine β-synthase (CBS) or cystathionine γ-lyase (CSE). If enough oxygen exists in the cell then H_2S is oxidized to sulfite and sulfate in the mitochondria or cytosol; however, if oxygen is limited H_2S accumulates and H_2S concentration increases in the cell (Olson, 2011). The pathways by which H_2S acts to cause a physiological response in the cell are still largely unknown but in mammals H_2S has been shown to act on several ion channels that have been proposed to be involved in hypoxia sensing in the carotid body (reviewed by Li et al., 2011) providing a link between the accumulation of this gasotransmitter and the hypoxic response.

8. Other neurotransmitters and neuromodulators

The NECs in fish gills have also been compared to the neuroepithelial bodies (NEBs) which are oxygen sensing cells located

Table 1
Summary of markers found in the NECs of fish, their distribution in the fish species studied to date and their proposed function related to the response to hypoxia.

Location	1° marker	Other markers	Proposed function	Species distribution	References
Filament	Serotonin	Synaptic vesicle	O_2 sensing	All fish species studied to date	Coolidge et al. (2008), Jonz et al. (2004), Jonz and Nurse (2003), Saltys et al. (2006), Burleson et al. (2002), and Brink and Milsom (unpublished)
Filament	Serotonin	Catecholamines	O_2 sensing	In channel catfish, but not trout and goldfish	Burleson et al. (2002) and Porteus et al. (in press)
Lamellae	Serotonin	Synaptic vesicle	Response to hypoxia	All slecies except trout and mangrove rivulus	Coolidge et al. (2008), Jonz et al. (2004), Jonz and Nurse (2003), Saltys et al. (2006), Burleson et al. (2002), and Brink and Milsom (unpublished)
Lamellae	Serotonin	Purinergic receptor	Unknown	Zebrafish	Jonz and Nurse (2003)
Filament	Synaptic vesicle	n/a	Response to hypoxia	Zebrafish, trout, goldfish, traira, trairaõ	Coolidge et al. (2008), Jonz et al. (2004), and Saltys et al. (2006)
Filament	Catecholamines	n/a	O_2 sensing	Channel catfish, not trout and goldfish[#]	Burleson et al. (2002) and Porteus et al. (in press)
Filament	Nitric oxide	n/a	Response to hypoxia	Channel catfish	Zaccone et al. (2003)

[#] See text for details.

Table 2
Summary of neural markers found in fish, their distribution in the fish species studied to date and their proposed function related to the hypoxic response.

Innervation	Location	1° marker	Other markers	Proposed function	Species distribution	References
Bipolar/chain neurons	Filament	Serotonin	Synaptic vesicle	Innervate NECs in filament and lamellae	Zebrafish	Jonz and Nurse (2003)
	Filament	Serotonin	Neuronal marker	Innervate NECs in filament and lamellae	Zebrafish	Jonz and Nurse (2003)
	Filament	Serotonin	Acetylcholine	Innervate NECs in filament and lamellae	Trout, goldfish	Porteus et al. (in press)
Extrinsic innervation	Filament and lamellae	Neuronal marker	n/a	Innervate NECs in filament	Zebrafish, trout, goldfish, traira, trairaõ	Jonz and Nurse (2003) and Coolidge et al. (2008)
	Filament and lamellae	Neuronal marker	Acetylcholine	Innervate NECs in filament	Trout, goldfish	Porteus et al. (in press)
Intrinsic innervation	Filament	Neuronal marker	n/a	Innervate NECs in filament only, some innervate the eFA		Jonz and Nurse (2003)
	Filament	Neuronal marker	Acetylcholine	Innervate NECs in filament only, some innervate the eFA	Trout, goldfish	Porteus et al. (in press)
eFA sphincter	Base of filament	Acetylcholine	n/a	Modulation of blood flow	Trout, eel, cod, perch	Bailly and Dunel-Erb (1986)
	Base of filament	Acetylcholine	SV2, zn-12	Modulation of blood flow	Trout, goldfish	Porteus et al. (in press)
	Base of filament	Catecholamines	n/a	Modulation of blood flow	Trout, perch	Dunel-Erb and Bailly (1986)
	Base of filament	Serotonin	n/a	Modulation of blood flow	Zebrafish	Jonz and Nurse (2003)
eFA walls	Filament	Nitric oxide	n/a	Involved in the hypoxic response	Channel catfish	Zaccone et al. (2003)

eFA: efferent filament artery.

in the lungs of mammals and birds which contain some regulatory peptides not found in the glomus cells (Adriaensen et al., 2006; Zaccone et al., 1997, 2006). Some of these peptides are leu-5-enkephalin, met-5-enkephalin and neuropeptide Y which are potent vasoconstrictors (Van Lommel et al., 1999). These peptides have been found in non-serotonin containing cells in various fish species (Goniakowska-Witalinska et al., 1995; Zaccone et al., 1992). The role of these cells in oxygen chemoreception remains unknown as studies directly linking these cells to the hypoxic response have not been undertaken.

9. Conclusions

Based on morphological, immunohistochemical and electrophysiological findings, fish gills contain multiple types of NECs. Those containing serotonin and/or catecholamines have been shown to depolarize in response to hypoxia although this evidence comes exclusively from studies of the large NECs found in the filaments of zebrafish and channel catfish (Burleson et al., 2002; Jonz et al., 2004). The extent to which any of the NECs containing other neurochemicals are sensitive to changes in O_2 remains to be determined as does the relative role of any of the NECs in the paracrine regulation of gill blood flow versus the reflex regulation of cardiorespiratory processes (Table 1). Certainly, the data collected to date suggest there may be significant species differences in the neurochemical profiles of both O_2 sensing NECs and innervation in fish gills and the bases for this remains unclear. It should be noted, however, that NECs in general are characterized by the presence of large, electron dense, cytoplasmic vesicles and are believed to be derived from APUD cells (amine precursor uptake and decarboxylation cells) descendent from the developing neural crest. In fish these cells have traditionally been characterized only by the presence of serotonin and their ability to label for one specific antibody for synaptic vesicles, SV2. In this regard it should be noted that cells that label for the vesicular ACh transporter do not label for SV2 although the ACh must be confined to synaptic vesicles. Furthermore, cells identified as serotonergic NECs do not label for the marker for migrating or proliferating neural crest cells, HNK-1 (Porteus et al., in press) indicating that either these are not derived from the neural crest, are only mature, derived cells, or that our markers lack specificity. This and other data presented in this review should serve as a strong reminder that most, if not all of the antibodies used for immunohistochemistry in fish are not derived from fish and thus lack of evidence should not necessarily be construed as evidence of absence. This may explain some of the variation seen in studies performed on different species and indicates that much work remains to be done to elucidate the full nature of the types and roles of NECs in chemosensing as well as their afferent and efferent innervation (summarized in Table 2) and the relative roles of the intrinsic versus extrinsic innervation.

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